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The Arabidopsis *SUPERMAN* Gene Mediates Asymmetric Growth of the Outer Integument of Ovules

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Arabidopsis *superman* (*sup*, also referred to as *floral mutant10*) mutants have previously been shown to have flowers with supernumerary stamens and reduced carpels as a result of ectopic expression of the floral homeotic gene *APETALA3* (*AP3*). Here, we report that *sup* mutations also cause specific alterations in ovule development. Growth of the outer integument of wild-type ovules occurs almost exclusively on the abaxial side of the ovule, resulting in a bilaterally symmetrical hoodlike structure. In contrast, the outer integument of *sup* mutant ovules grows equally on all sides of the ovule, resulting in a nearly radially symmetrical tubular shape. Thus, one role of SUP is to suppress growth of the outer integument on the adaxial side of the ovule. Genetic analyses showed that the effects of *sup* mutations on ovule development are independent of the presence or absence of AP3 activity. Thus, SUP acts through different mechanisms in its early role in ensuring proper determination of carpel identity and in its later role in asymmetric suppression of outer integument growth.

INTRODUCTION

Flowers of angiosperms can be considered to include five types of organs. Four sets of these organs—sepals, petals, stamens, and carpels—develop in concentric whorls or spirals directly from the floral apex. In contrast, the fifth set of organs, the ovules, develop inside the carpels from specialized meristematic regions, the placentas. Angiosperm ovules are compound organs that commonly include four distinct morphological substructures—the funiculus (a supporting stalk), a terminal nucellus, and two integuments that enclose the nucellus. The nucellus is the site of meiotic production of megaspores and of subsequent development of one or more of these megaspores into a megagametophyte (embryo sac), which includes the egg cell. Following fertilization, the ovule develops into a seed.

Genetic analyses of flower development in Arabidopsis and Antirrhinum have led to the formulation of models for genetic determination of the identity of the four whorls of floral organs in these two species (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991b; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991). Several current reviews summarize the results of these studies showing that specific combinations of the products of at least five different floral homeotic genes regulate the identity of these organs (Okamuro et al., 1993; Ma, 1994; Weigel and Meyerowitz, 1994). More recently, work in our laboratory and other laboratories has provided initial information on the nature and function of genes regulating ovule development in Arabidopsis. An initial description of two mutations affecting Arabidopsis ovule development, *short integuments* (*sin1*) and *bell* (*bel1*) (Robinson-Beers et al., 1992), has been followed by more detailed analyses of the functions of both of these genes (Lang et al., 1994; Modrusan et al., 1994; Ray et al., 1994). *aberrant testa shape* (*ats*), another mutation that affects ovule development, leads to the formation of seeds with altered shapes (León-Kloosterziel et al., 1994). Reiser and Fischer (1993) have briefly described two female-sterile mutants, *ovm2* and *ovm3*, that have alterations in embryo sac and integument formation, respectively.

The action of genes controlling ovule morphogenesis is not completely independent of the genes responsible for determining the identity of the four whorls of floral organs. For example, ovules form from the surface of carpels, and carpel formation is normally directed by expression of *AGAMOUS* (*AG*), one of the floral organ identity genes (Bowman et al., 1991a, 1991b; Meyerowitz et al., 1991; Meyerowitz, 1994). The initiation of ovule development is therefore normally dependent on *AG* expression. Recent work shows, however, that following ovule initiation, *AG* expression must be suppressed in the ovule primordia for normal manifestation of the remainder of the ovule development program and that the *BEL1* gene is an essential component of this suppression (Modrusan et al., 1994; Ray et al., 1994). Absence of BEL1 activity in *bel1* mutants leads to continued expression of *AG* in the ovules (Modrusan et al., 1994; Ray et al., 1994), resulting in homeotic transformation of integuments into carpels (Ray et al., 1994). Thus, ovule development is normally dependent both on appropriate activation of *AG* expression and on subsequent suppression of *AG*

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expression by BEL1. In this role, *BEL1* is acting as a "cadastral" gene; this type of gene defines the spatial expression pattern of other genes.

In this study, we show that another gene previously known only to be involved in determination of carpel identity, *SUPERMAN* (*SUP* [Meyerowitz et al., 1991], also referred to as *FLORAL MUTANT10* [*FLO10*; Schultz et al., 1991]), plays an additional role in direct regulation of ovule development. Previous work with this gene has shown that *SUP* is a cadastral gene that negatively regulates expression of another member of the set of organ identity genes, *APETALA3* (*AP3*), in the fourth floral whorl. *AP3* is normally expressed only in the second and third whorls of floral organs, where it contributes to determination of the identity of petals and stamens (Jack et al., 1992; Goto and Meyerowitz, 1994). In *sup* mutants, the zone of *AP3* expression expands into the region of the floral apex from which the fourth whorl carpels normally develop, resulting in a complete or partial conversion of the carpels into stamens (Meyerowitz et al., 1991; Schultz et al., 1991; Bowman et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994). These previous studies with *SUP* addressed only the effects of *sup* mutations on the identity of the four whorls of floral organs. Here, we report the isolation of a new allele of *sup* (*sup-5*) and show that, in addition to effects on carpel identity, this mutation and other mutations at the *sup* locus cause specific alterations in the development of the outer integuments of ovules. We further demonstrate that the role of *SUP* in normal ovule development is independent of its cadastral role in regulating the pattern of *AP3* and *PISTILLATA* (*PI*) expression.

RESULTS

Identification of the *sup-5* Mutant

In a screen for mutants with reduced female fertility, a novel mutant with abnormal ovules and supernumerary stamens was isolated. This mutant, which had apparently normal pollen but reduced seed set (averaging less than two seeds per fruit) was initially referred to as *hairdryer* (*hai*) because of the shape of the ovules. The floral phenotype of *hai* mutants was similar to that resulting from mutations at a described locus, which has been referred to as either *SUP* (Bowman et al., 1992) or *FLO10* (Schultz et al., 1991). Seeds homozygous for the *sup-1* allele were obtained to determine possible allelism between the *SUP* locus and the newly isolated *hai* ovule mutant. Because both of these mutants have defective pistils, pollen from a *sup-1*/*sup-1* plant was used to pollinate pistils of plants that were heterozygous for *hai*. The F_1 progeny from four crosses consisted of 21 wild-type plants and 20 plants with a Sup^- phenotype, indicating that half the progeny were heterozygous for *sup-1* and half were heteroallelic for *sup-1* and *hai*. This 1:1 segregation of mutant to wild type confirms that *hai* is allelic to *sup-1* (and *flo10*). Because this is the fifth allele described for this locus, we designate this new allele *sup-5* (or *flo10-5*) and use the *sup-5* designation throughout the text.

sup Mutants Have Abnormal Ovules

Previous reports of *sup* mutants (Schultz et al., 1991; Bowman et al., 1992) addressed only the effects of this mutation on the four whorls of floral organs. These studies showed that the phenotype of *sup* mutants consists of supernumerary stamens and absent or aberrant carpels. The nature of the ovules of *sup* mutants was not addressed in either of the previous studies. Here we present the results of a detailed characterization of *sup-5* ovules and show that they are abnormal and morphologically indistinguishable from those of the previously described *sup-1* mutant (Bowman et al., 1992).

Morphological development of Arabidopsis ovules has been described in detail (Robinson-Beers et al., 1992; Modrusan et al., 1994) and is only briefly reviewed here. Ovule primordia first arise from the carpel wall as undifferentiated protuberances at stage 9 of flower development (flower stages are from Smyth et al., 1990). These protuberances elongate during stage 10, and two integuments are initiated during stage 11. The integument primordia demarcate the border between the terminal nucellus and supporting funiculus (Robinson-Beers et al., 1992). As illustrated in Figure 1A, the two integument primordia differ morphologically even at the earliest developmental stages. The inner integument arises as a symmetric collar of tissue encircling the funiculus (Figure 1A) and continues to develop symmetrically as it elongates to envelop the nucellus (Robinson-Beers et al., 1992). In contrast, the outer integument primordium is asymmetrical due to a greater number of cell divisions on one side of the ovule primordium relative to the other (Figure 1A; Robinson-Beers et al., 1992). The side of the ovule primordium on which the larger number of divisions occurs is oriented toward the base of the carpel and is therefore referred to as the abaxial side of the ovule. On the opposite or adaxial side of the ovule (oriented toward the upper, stigmatic end of the carpel), the outer integument primordium exhibits fewer cell divisions (Figure 1A).

During stage 12, further development of the outer integument continues this same growth pattern, exhibiting a gradient of cell division and expansion around the circumference of the ovule, with maximal growth of the outer integument on the abaxial side of the ovule and essentially no growth on the adaxial side (Figure 1A; Robinson-Beers et al., 1992). By stage 13 (anthesis), as a result of this asymmetric growth, the outer integument is a highly curved hoodlike structure that completely encloses both the inner integument and the nucellus (Figure 1B). Concomitant with the growth of the integuments, the cells of the funiculus divide, and the funiculus curves in the direction opposite that of asymmetric outer integument growth. The combination of funicular curvature and asymmetric outer integument growth leads to the bilaterally symmetrical, S-shaped and amphitropous configuration characteristic of Arabidopsis ovules (Robinson-Beers et al., 1992).

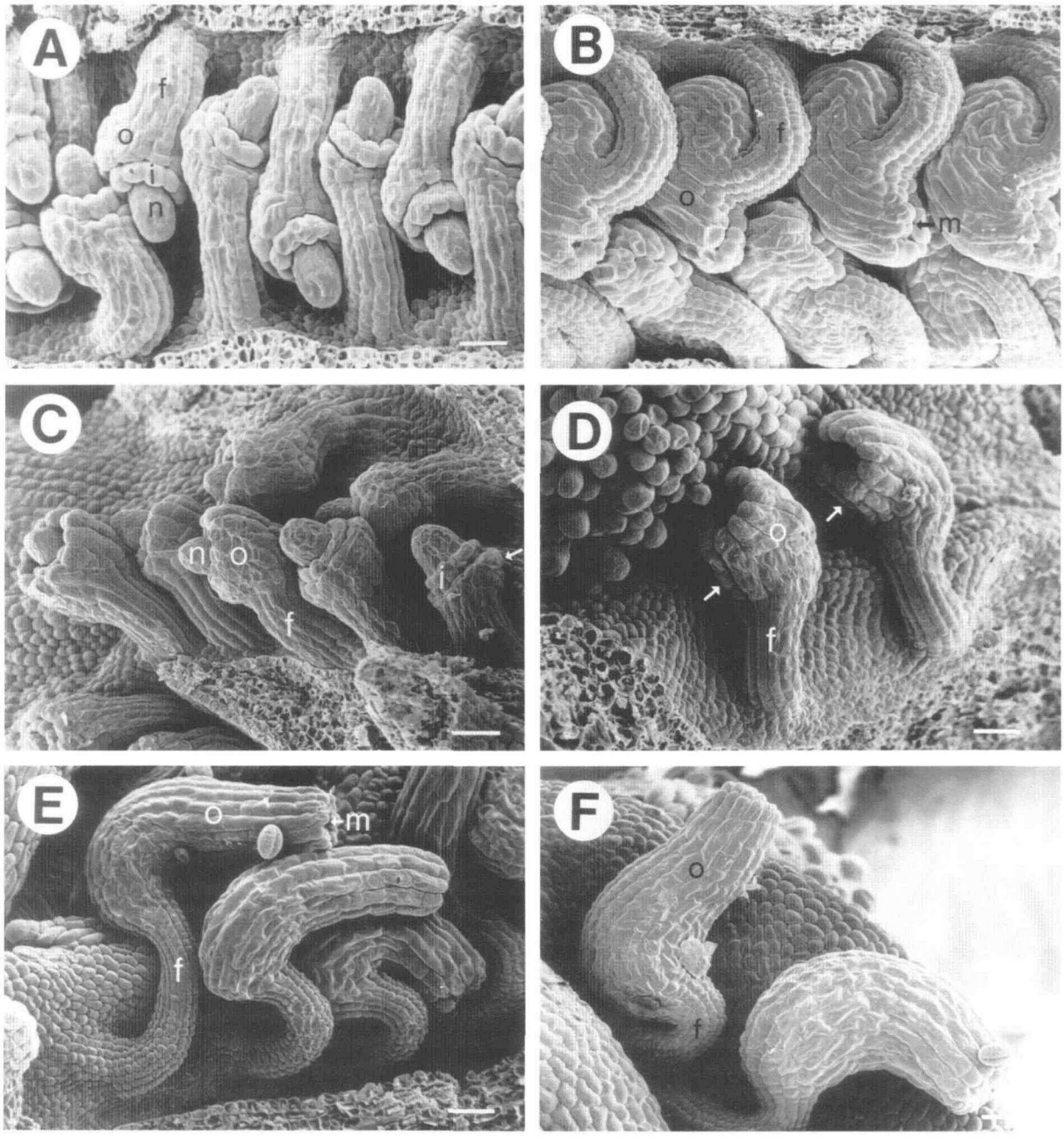

Figure 1. Scanning Electron Micrographs of Ovule Development in Wild-Type Arabidopsis and *sup* Mutants.

(A) Wild-type ovules at early stage 12. The asymmetric shape of the outer integument is well established by this stage. The abaxial side of the ovules (and hence the base of the pistil) is to the left. Bar ≈ 20 μm.

(B) Mature wild-type ovules at stage 13 (anthesis). The outer integument has expanded to enclose both the inner integument and the nucellus. The abaxial side of the ovules is to the left. Bar = 30 μm.

(C) *sup-5* ovules at early stage 12. Note that ovule development in Arabidopsis is asynchronous, and in this image a developmental series can be seen from least mature (at right) to more mature (at left). The least mature ovule, at the extreme right, is nearly wild type in appearance, having an outer integument primordium that appears normal (arrow). Aberrant elongation of the adaxial surface of the outer integument, which is characteristic of *sup* mutants, is apparent in the other ovules that are further developed. The abaxial side of the ovules is to the right. Bar = 20 μm.

(D) *sup-5* ovules at late stage 12. The adaxial surfaces of the outer integuments (arrows) have elongated to the same extent as the abaxial surfaces. The abaxial side of the ovules is to the right. Bar = 20 μm.

(E) *sup-5* ovules at stage 13. The range of *sup* ovule phenotypes is shown. The abaxial side of the ovules is to the left. Bar = 30 μm.

(F) *sup-1* ovules at stage 13. The abaxial side of the ovules is to the left. Bar = 30 μm.

f, funiculus; i, inner integument; m, micropyle; n, nucellus; o, outer integument.

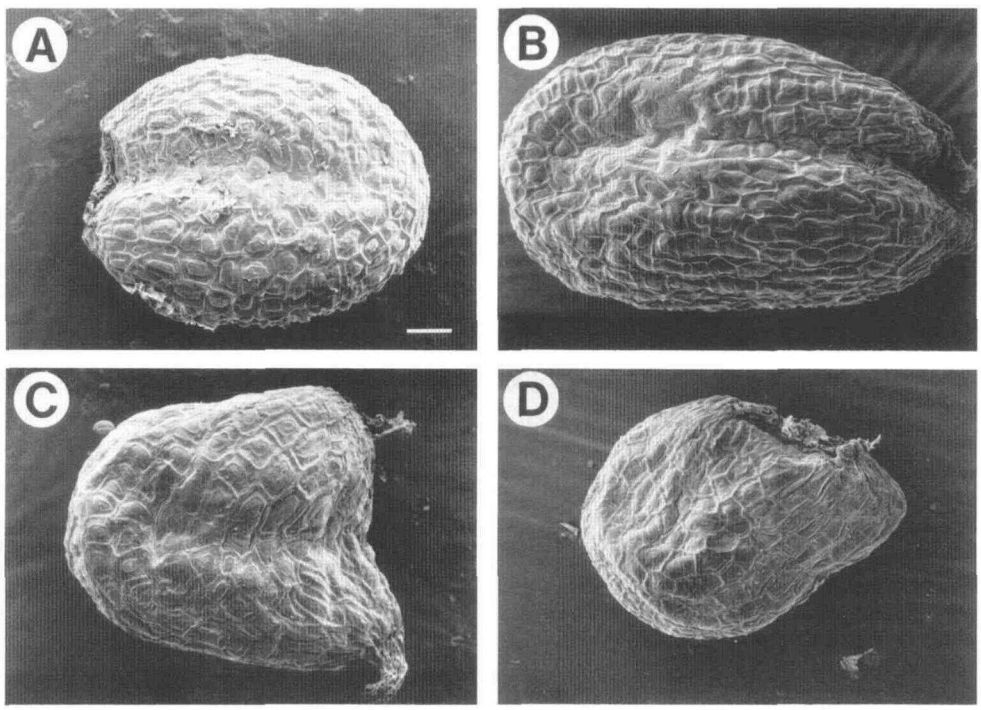

Figure 2. Scanning Electron Micrographs of Wild-Type and *sup* Seeds.

(A) Wild type.
(B) *sup-1.* Near wild type.
(C) *sup-1.* Heart shape.
(D) *sup-1.* Globular shape.
Bar in **(A)** = 50 μm for **(A)** to **(D)**.

Early ovule development in *sup* mutants, up to and including the formation of a symmetrical inner integument primordium and an asymmetrical outer integument primordium, is indistinguishable from development of wild-type ovules (Figure 1C). However, as the integuments extend during stage 12, the outer integument of *sup* ovules grows equally around the circumference of the nucellus (Figure 1C) rather than asymmetrically as in wild-type ovules. In this respect, growth of the outer integument of *sup* mutants resembles that of the inner integument of wild-type ovules. The result of symmetric growth of the outer integument is that *sup* ovules at stage 12 have a decidedly less curved appearance than wild-type ovules at the same stage (compare Figure 1A with Figures 1C and 1D). By anthesis (stage 13), *sup* ovules are strikingly abnormal in appearance due to the elongated, tubular shape of the outer integument (compare Figures 1B and 1E). Counting the number of cells and measuring the length of the abaxial surface of the outer integument in photomicrographs indicated that this side of *sup-5* ovules did not differ in number of cells or overall length from the abaxial surface of wild-type ovules (data not shown). In contrast, the adaxial surface of *sup-5* ovules is clearly much longer than that of the wild type (Figures 1B and 1E). The abnormal morphology of the *sup-5* ovules therefore results primarily (or exclusively) from aberrant growth of the adaxial side of the outer integument. We could discern no difference in ovule morphology between *sup-5* and the previously identified *sup-1* mutant at anthesis (Figures 1E and 1F) or at any earlier stage of development (data not shown), demonstrating that the *sup* ovule phenotype is a result of lesions in the *SUP* locus and is not a special property of our *sup-5* isolate.

Even within a single carpel, there was some variability in the phenotype of *sup* ovules. The three ovules shown in Figure 1E range from the most severe (and most common) phenotype in which the ovule resembles an inverted L to the least severe phenotype in which the ovule superficially has the wild-type amphitropous configuration but still exhibits symmetric growth characteristic of the outer integument of *sup* mutants. This variability is reflected in the phenotypes of the relatively small numbers of seeds recovered from homozygous *sup* mutants (100 to 200 per plant for *sup-5* versus ~4000 per plant for the wild type). Figure 2 shows a wild-type seed and three *sup* seeds, illustrating the most common seeds of a set of widely varying morphologies. Phenotypically Sup^- seeds are produced by *sup* plants even when these plants have been

pollinated with wild-type pollen (data not shown). This demonstrates that it is the maternal genotype rather than that of the embryo that determines the morphology of these seeds. Again, no differences were observed between the seeds of *sup-1* and *sup-5* mutants, although *sup-1* plants produced even fewer seed than *sup-5* plants.

The Inner Integument and Embryo Sac of *sup* Mutants

To examine the effects of *sup* mutations on internal structures of ovules, clearings (Herr, 1971) of wild-type, *sup-1*, and *sup-5* pistils were prepared. Figure 3 shows ovules dissected from the cleared pistils and viewed using differential interference contrast light microscopy. The dramatic increase in the length of the adaxial side of the outer integument in the Sup$^-$ ovule (Figure 3B) relative to that of the wild-type ovule (Figure 3A) is readily apparent in this view. In contrast, in both wild-type and Sup$^-$ ovules, the abaxial and adaxial sides of the inner integument extend only far enough to completely enclose the embryo sac. Thus, although the inner integument of *sup* ovules is somewhat less curved than that of wild-type ovules, the inner integument of *sup* mutants is less severely altered than is the outer integument. No differences between ovules of *sup-1* and *sup-5* mutants were observed (data not shown).

sup-5 Flowers Are Not as Severely Altered as Those of *sup-1*

In contrast to the ovule phenotype, the phenotype of *sup-5* flowers differs from that of *sup-1* flowers. As previously described (Schultz et al., 1991; Bowman et al., 1992), mutations at the *sup* (*flo10*) locus result in the formation of extra stamens interior to the third whorl of organs. Concomitant with excess stamen production is a decrease in pistil tissue, with mosaic organs of stamen and carpel tissue common at the center of the flower. The pistil phenotypes for *sup-1* (and *flo10*) mutants ranged from an absence of any discernible pistil structure in the most extreme case to flowers with nearly wild-type pistils with patches of stamen tissue present (Schultz et al., 1991; Bowman et al., 1992). A detailed comparison of *sup-1* and *sup-5* flower morphology was made by dissecting flowers at anthesis (stage 13) from seven inflorescences of each genotype and evaluating each flower in terms of the number of stamens, presence or absence of ovules, and pistil type. To eliminate differences in pistil type due to developmental stage, only flowers at anthesis were examined.

Pistils were categorized into five types similar to those identified by Bowman et al. (1992). Figure 4 illustrates some of these pistil types, which are defined as follows: none, no recognizable pistil structure or differentiated pistil tissue (Figure 4A); filament, a filamentous structure capped by stigmatic tissue (Figure 4B); stamen/carpel, a mosaic organ composed of both carpelloid and staminoid tissues and open on at least one side (Figure 4C); closed stamen/carpel, a closed cylinder resembling a wild-type pistil but including staminoid tissue (data not shown); and wild type, a closed cylinder resembling a wild-type pistil and including no visible staminoid tissue (data not shown). Variability in the stamen/carpel category made this grouping the broadest because it was often difficult to assess the relative degree of staminoidy or carpelloidy in each flower.

The data in Table 1 indicate that flowers of *sup-5* mutants are less severely altered than are those of *sup-1* mutants. The number of stamens in *sup-5* flowers is closer to that of the wild type, and the conversion of the fourth whorl organs into staminoid structures is much less severe in *sup-5* than in *sup-1*. Thus, with respect to the flower phenotype, *sup-5* appears a weaker allele than *sup-1*. However, as previously described, we were unable to detect differences between *sup-1* and *sup-5* ovules. This provides an initial indication that control of staminoidy in the fourth whorl is in some respects separate from the influence of SUP on ovule development.

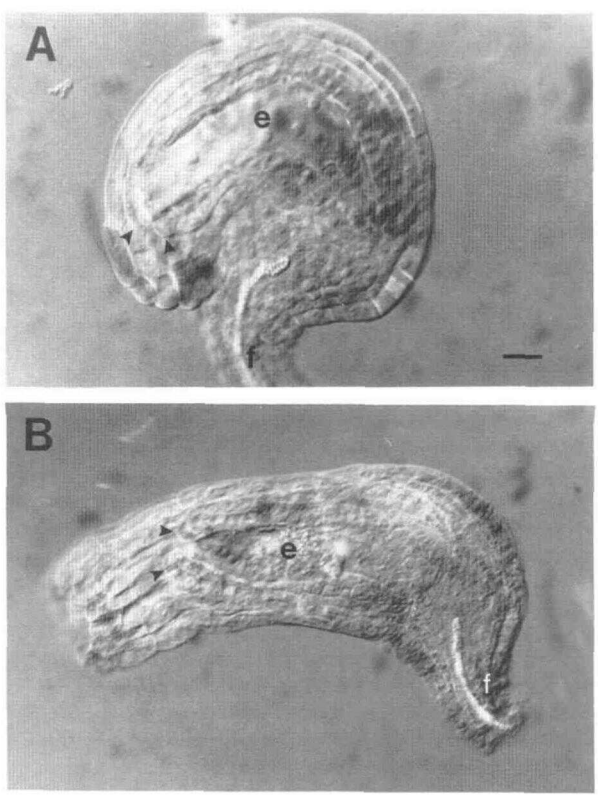

Figure 3. Light Micrographs of Cleared Ovules Viewed with Differential Interference Contrast Optics.

(A) Wild type.
(B) *sup-5*.
Bar in **(A)** = 15 μm for both **(A)** and **(B)**. e, embryo sac; f, funiculus; arrowheads indicate the micropylar edges of the inner integuments.

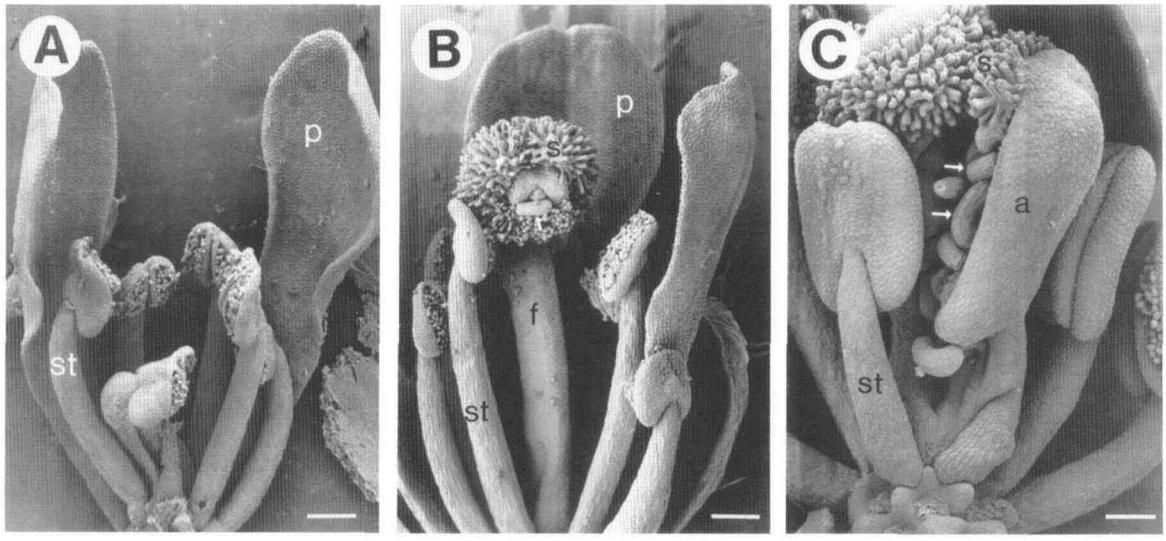

Figure 4. Pistil Types Used for Comparison of *sup-1* and *sup-5* Flowers.

(A) No pistil tissue (none): *sup-1* flower at stage 13 that completely lacks ovules and an organized pistil structure. Four sepals, two petals, and three stamens were removed to reveal the fourth whorl. Bar = 20 μm.

(B) Filament: *sup-1* flower at stage 13 exhibiting a pistil composed of a filamentous structure capped by stigmatic tissue. Three sepals, two petals, and three stamens were removed to reveal the fourth whorl. The arrow indicates a Sup⁻ ovule. Bar = 50 μm.

(C) Stamen/carpel: *sup-1* flower at stage 13 with a mosaic pistil composed of stamen and carpel tissue. Four sepals, three petals, and four stamens were removed to reveal the inner whorls. Note the anther-like structure bearing Sup⁻ ovules (arrows) and capped by stigmatic tissue. Bar = 120 μm.

p, petal; st, stamen; f, filamentous structure; a, anther-like structure; s, stigmatic tissue.

Double Mutant Analysis

AP3 *Does Not Play a Role in the Ovule Phenotype of* sup *Mutants*

Previous reports describing mutations at the *SUP* locus (Schultz et al., 1991; Bowman et al., 1992) have demonstrated that the production of stamens in the fourth whorl of floral organs in *sup* flowers is associated with ectopic expression of the floral homeotic gene *AP3* in this region. Further, in transgenic plants harboring an *AP3* gene under the control of the cauliflower mosaic 35S promoter, Jack et al. (1994) observed a similar increase in stamen number and a concomitant decrease in pistil tissue resulting from the engineered ectopic expression of *AP3*. This indicates that ectopic *AP3* expression is sufficient to explain this aspect of the *sup* mutant phenotype. Thus, it was possible that ectopic *AP3* expression in the gynoecia of *sup* mutants was also responsible for aberrations in ovule development. To test this hypothesis, we constructed and examined double mutants homozygous for both *sup* and *ap3* mutations. Because the ovule phenotypes of different *sup* mutants were indistinguishable and because the *sup-5* mutant more frequently produced flowers that contained ovules (Table 1), the *sup-5* allele was used in these studies.

Figure 5 shows reproductive structures of F_2 progeny resulting from the pollination of *ap3-3* flowers with *sup-5* pollen. This population segregated for four distinct phenotypes in a 9:3:3:1 ratio (238:76:69:16, $\chi^2 = 4.608$, $P > 0.20$): plants with wild-type flowers and wild-type ovules (data not shown), plants with *ap3-3* flowers and wild-type ovules (Figure 5A), plants with *sup-5*

Table 1. Comparison of *sup-1* and *sup-5* Flower Morphology

		Pistil Type[a] (%)					
Mutation	Stamens/Flower	None	Filament	Stamen/ Carpel	Closed	Wild Type	% Flowers with Ovules
sup-1	8.8 (± 0.3)[b]	26	16	56	0	1	70
sup-5	7.3 (± 0.1)	0	1	62	30	7	100

[a] See text.
[b] Mean (± SE).

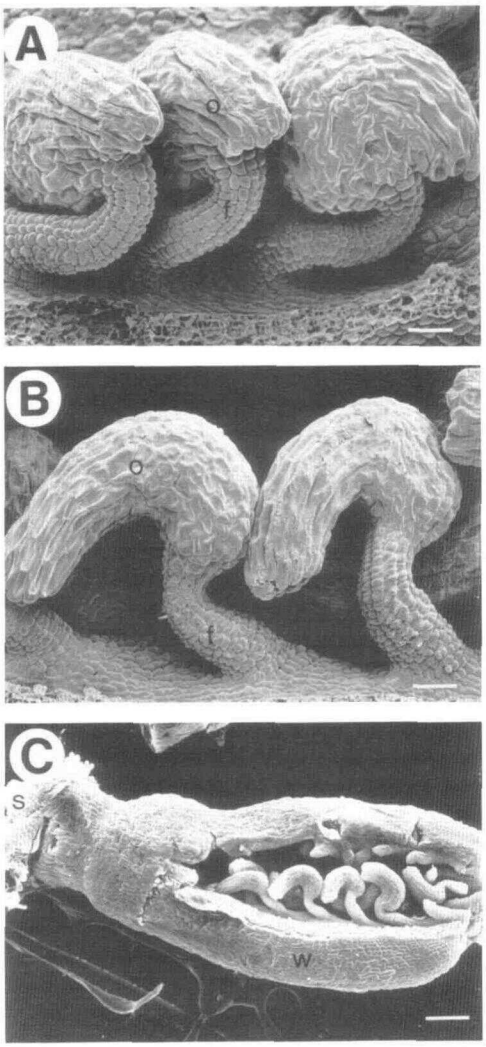

Figure 5. Analysis of Interactions of *sup-5* and *ap3-3* Mutations.

(A) *ap3-3* ovules at stage 13. Bar = 25 μm.
(B) *ap3-3 sup-5* double-mutant ovules at stage 13. Bar = 25 μm.
(C) *ap3-3 sup-5* double-mutant pistil at stage 13. One of four locules of the compound pistil (consisting of organs from both the third and fourth whorls) is opened to show Sup⁻ ovules. Bar = 100 μm.
f, funiculus; o, outer integument; s, stigma; w, ovary wall.

flowers and *sup-5* ovules (data not shown), and plants with *ap3-3* flowers and *sup-5* ovules (Figures 5B and 5C). The ratios and the nature of the phenotypes of these classes indicate that they represent plants not homozygous for either mutation, plants homozygous only for *ap3-3*, plants homozygous only for *sup-5*, and plants homozygous for both *ap3-3* and *sup-5*, respectively. The overall floral morphology of *sup-5 ap3-3* double mutants was indistinguishable from that of *ap3-3* single mutants (data not shown, but see Jack et al., 1992). That is, these flowers had normal sepals in the first whorl and sepaloid petals in the second whorl. Stamens were not observed in the third whorl, but filamentous structures without anthers were occasionally present. The central part of the flowers consisted of a compound pistil, usually comprising four fused carpels (Figure 5C), rather than the two fused carpels characteristic of wild-type Arabidopsis. This feature of the *ap3-3* phenotype is the result of conversion of third whorl organs to carpels (Jack et al., 1992). Ovules of the double mutants were indistinguishable from those of *sup-5* single mutants (compare Figure 1E to Figure 5B). This demonstrates that the presence of an active *AP3* gene is not required for the formation of phenotypically Sup⁻ ovules.

Previous studies examined the effects of these two mutations only on third and fourth whorl organs. In these studies, F_2 populations resulting from crosses between *sup* and *ap3* mutants exhibited a 9:3:4 ratio of phenotypically wild type, Sup⁻, and Ap3⁻ plants, respectively (Schultz et al., 1991; Bowman et al., 1992). Using these same criteria, our data produced a similar 9:3:4 ratio (238:76:85, $\chi^2 = 3.138$, $P > 0.20$).

Altered Ovules in sup *Mutants Contribute to Female Infertility*

sup mutations cause both severe aberrations in the structure of the pistil and production of aberrantly shaped ovules. Either or both of these effects could be the cause of the dramatically reduced female fertility of *sup* mutants. In contrast, *ap3-3 sup* double mutants have relatively normal pistils (which consist of a larger number of carpels than those of normal flowers) and ovules that are indistinguishable from those of *sup* single mutants. As a partially independent test of the relative effects of the carpel and ovule phenotypes of *sup* mutants on female fertility, we compared the seed set of the three classes of mutants in the populations segregating for *sup-5* and *ap3-3*. Flowers of plants that were phenotypically Sup⁻, Ap3⁻, and both Sup⁻ and Ap3⁻ were pollinated with stamens isolated from wild-type Landsberg *erecta* plants. Because we were assessing maximal seed set, those flowers that had stamens were not emasculated before pollination. Pistils of wild-type flowers that were allowed to self-pollinate were also compared. The results of these experiments are shown in Table 2.

The data in Table 2 show that the fraction of pollinated pistils that expanded in *sup-5* mutants was significantly lower (99% confidence limit) than that of *sup-5 ap3-3* double mutants. However, when individual ovules were examined in the developing siliques, the fraction of ovules that developed into seeds was similar between these two phenotypic classes but significantly lower than the frequencies for seed set in either *ap3-3* single mutants or wild-type plants. The only apparent difference between the *ap3-3* single mutants and the *ap3-3 sup-5* double mutants is the Sup⁻ phenotype of the ovules of the latter. Thus, the nature of *sup-5* ovules appears to reduce the frequency of successful fertilization, even in plants with similar pistil morphologies. The even lower frequency of pistils containing developing seed in *sup-5* single mutants, relative to *ap3-3 sup-5* double mutants, indicates that the aberrant pistil

Table 2. Seed Set in *ap3-3*, *sup-5*, and *ap3-3 sup-5* Mutant Plants

Genotype	% of Fertilized Pistils That Expand	% of Ovules Forming Seeds in Expanded Pistils	Mean Number of Ovules/Pistil (± SE)	Mean Number of Seeds/Expanded Pistil (± SE)
sup-5	28.6[a] (13.4 to 53.2[b])	17.8 (12.2 to 26.6)	25.9 (± 6.6)	6.3 (± 5.0)
ap3-3	82.4 (50.5 to 96.1)	48.9 (40.8 to 59.1)	35.8 (± 13.0)	19.9 (± 9.6)
sup-5 ap3-3	84.4 (70.0 to 98.9)	16.8 (14.1 to 20.3)	44.8 (± 9.2)	8.9 (± 4.8)
Wild type	100.0 (76.7 to 100)	76.9 (71.8 to 81.7)	51.0 (± 4.2)	39.2 (± 4.0)

[a] Actual percentages.
[b] 99% confidence limits according to binomial distribution.

phenotype of Sup⁻ plants also contributes to a decrease in seed set.

sup *Mutations Do Not Alter the Integument-like Structure Present in* bel1-1 *Mutants*

As illustrated in Figure 6A, mutations at the *BEL1* locus result in ovules with a single integument-like structure in place of the inner and outer integuments (Robinson-Beers et al., 1992; Modrusan et al., 1994). The integument-like structure of *bel1-1* ovules ceases to develop after forming a collar-shaped structure, which rarely envelops the nucellus at anthesis (Robinson-Beers et al., 1992; Figure 6A). *bel1-1 sup-5* double mutants were easily recognized as plants with Sup⁻ flowers bearing Bel1⁻ ovules (Figure 6C). The ovules of the double mutants were indistinguishable from those of *bel1-1* single mutants (compare Figures 6A and 6B). This result indicates that the effect of *sup* mutations on ovule development takes place after the establishment of the integument primordia and that the subsequent development of the integument-like structure in *bel1* mutants is not influenced by mutations at the *SUP* locus. It is not surprising that *bel1-1* has no influence on the pistil structure or stamen number of double-mutant flowers because there is no evidence of *BEL1* influence on flower morphology in *bel1* single mutants (Robinson-Beers et al., 1992; Modrusan et al., 1994).

Effects of the inner no outer *Mutation on Ovules Are Epistatic to* sup

Arabidopsis plants homozygous for the *inner no outer* (*ino*) mutation produce ovules that lack outer integuments (K. Robinson-Beers and C.S. Gasser, manuscript in preparation). The outer integument primordium is formed on ovules of *ino* mutants but does not develop further. As a result, *ino* mutant ovules at anthesis have only inner integuments and do not adopt the amphitropous configuration of wild-type ovules (Figure 6D). The inner integument of *ino* mutants appears normal, but it is difficult to ascertain all possible effects on the inner integument that may result from lack of an enclosing outer integument.

Because the inner integument is visible in *ino* mutants, any alteration of this structure that resulted from *sup* mutations should be evident in *ino sup-5* double-mutant ovules. A population segregating for both *ino* and *sup-5* mutations was found to include plants with Sup⁻ gynoecia bearing Ino⁻ ovules (Figure 6F). A closer view of the ovules of these double mutants shows that the ovules are indistinguishable from those of *ino* single mutants (compare Figures 6D and 6E). This result supports the hypothesis that mutations at the *SUP* locus specifically alter the growth asymmetry of the outer integument and do not directly affect the inner integument. In other aspects of floral morphology, *ino sup-5* double mutants display the Sup⁻ phenotype (Figure 6F). This result is expected in light of the wild-type flower morphology of *ino* mutants (K. Robinson-Beers and C.S. Gasser, manuscript in preparation).

DISCUSSION

SUPERMAN Is Required for Normal Development of the Outer Integument

Previous phenotypic descriptions of plants with mutations at the *SUP* (*FLO10*) locus have focused on the effects on stamen number and the degree of stamen-to-pistil conversion in the fourth whorl (Schultz et al., 1991; Bowman et al., 1992). In this study, we report that *sup* mutants also exhibit aberrant ovule morphology as a result of specific alterations in the development of the outer integument. The *SUP* gene does not appear to be required for the early stages of ovule development because wild-type and *sup* ovules are identical through the time of formation of the outer integument and inner integument primordia into early stage 12. However, during mid- to late-stage 12, the Sup⁻ phenotype becomes apparent as the region of the outer integument on the adaxial side of *sup* mutant ovules begins to grow at the same rate as the region on the abaxial side (Figures 1C and 1D). This aberrant growth results in ovules that have an elongated, tubular appearance at anthesis (Figure 1E) and that lack the amphitropous ovule morphology characteristic of Arabidopsis ovules (Figure 1B).

The inner integument of *sup* mutants is similar in length to that of wild-type ovules and does not extend significantly beyond the micropylar end of the embryo sac but is less curved than that of wild-type ovules (Figure 3). Another ovule mutant, *ino*, which does not have an outer integument, exhibits a similar

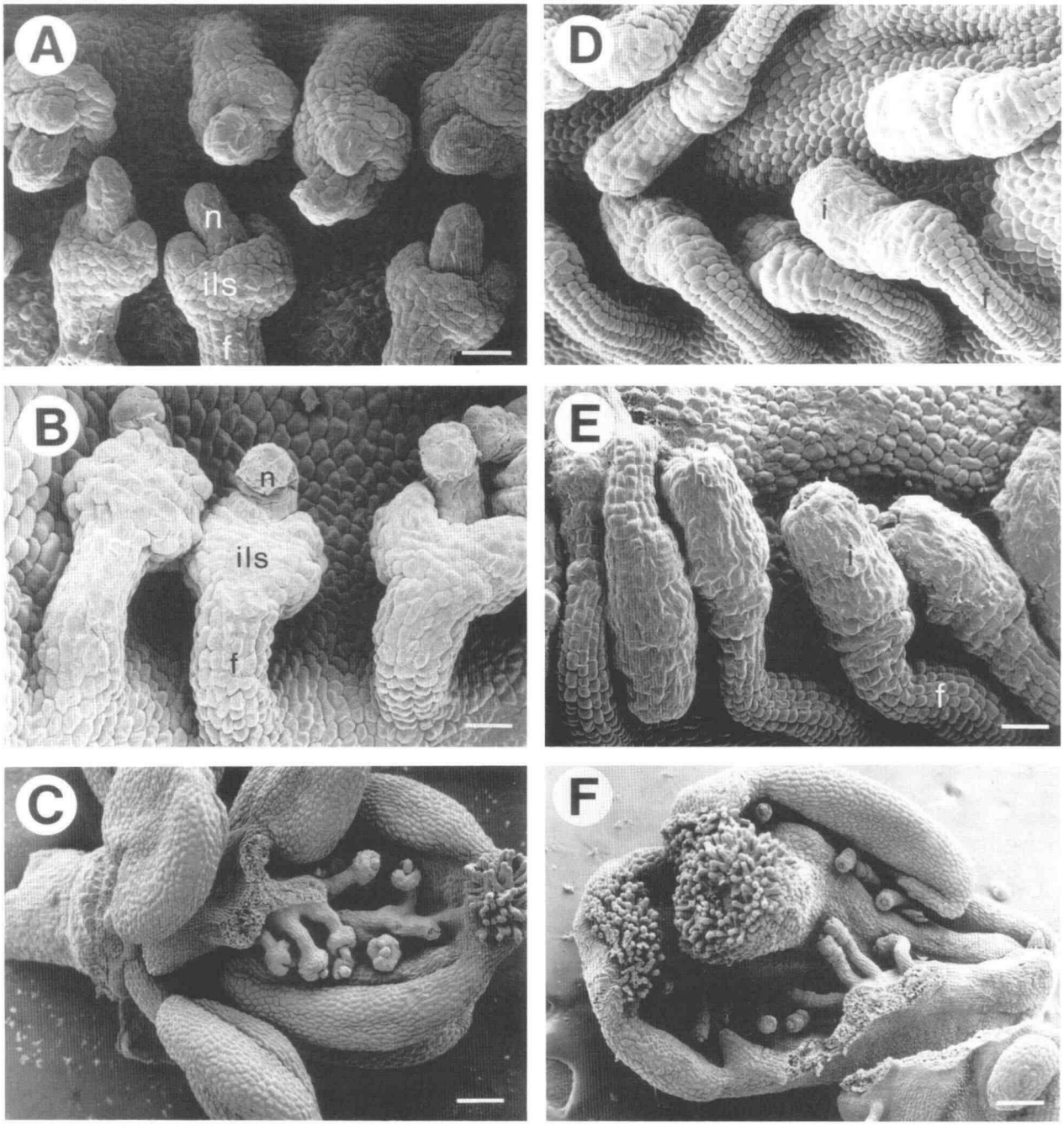

Figure 6. Analysis of Interactions between *sup-5* and *ino* and *bel1-1* Mutations.

(A) *bel1-1* ovules at stage 13. Bar = 16 μm.
(B) *bel1-1 sup-5* ovules at stage 13. Bar = 16 μm.
(C) Typical pistil from stage 12 *bel1-1 sup-5* double-mutant flower; note the Bel1$^-$ ovules in mosaic stamen/carpel Sup$^-$ pistil. Bar = 100 μm.
(D) *ino* ovules at stage 13. Bar = 26 μm.
(E) *ino sup-5* ovules at stage 13. Bar = 26 μm.
(F) Typical pistil from stage 12 *ino sup-5* double-mutant flower; note Ino$^-$ ovules in mosaic stamen/carpel Sup$^-$ pistil. Bar = 100 μm.
f, funiculus; i, inner integument; ils, integument-like structure (of *bel1* mutants); n, nucellus.

lack of curvature in the inner integument. This indicates that inner integument curvature may be an indirect result of confinement within an asymmetrically growing outer integument. In addition, the inner integument of *ino sup-5* double-mutant ovules is indistinguishable from that of *ino* single mutants (Figures 6D and 6E). These observations indicate that *sup* mutations do not directly affect inner integument development. Thus, it appears that the role of the *SUP* gene in ovule development is specifically to repress growth of the outer integument on the adaxial side of the ovule to produce the normal bilateral symmetry characteristic of the Arabidopsis outer integument.

As noted previously, the morphology of the integument primordia is not altered in *sup* mutants. Rather, it appears that *sup* mutations only affect the subsequent asymmetric growth of the outer integument, indicating that these two stages of outer integument formation are, at least in part, under separate control. A similar separation between the initial anatomy of primordia and their subsequent development has been noted in Arabidopsis homeotic mutants that exhibit altered development of the whorled floral organs (Hill and Lord, 1989; Crone and Lord, 1994). Thus, separation between regulatory programs for initial phases of primordium development and for subsequent development of mature organs may be a common phenomenon in plant development.

In *bel1-1* ovules, an integument-like structure replaces both of the integuments (Figure 6A). The integument-like structure has been shown to represent a deviation from integument identity in that it can go on to form a complete carpel (Modrusan et al., 1994; Ray et al., 1994). This homeotic alteration appears to result from ectopic expression of *AG* (Ray et al., 1994). The integument-like structure of *bel1-1 sup-5* double-mutant ovules is indistinguishable from that of *bel1-1* single mutants (Figure 6B). This indicates that *bel1* mutations alter the identity of the integument primordia prior to the steps governed by the *SUP* gene and that *BEL1* is epistatic to *SUP* with respect to the ovule but not the floral phenotype.

The small number of successful fertilization events that occur in *sup* mutants result in the formation of abnormally shaped seeds (Figure 2). Variability in the shapes of these seeds reflects the variability in the overall shape of *sup* ovules. The previously described *ats* mutant, which also produces ovules with aberrant integuments, was also found to produce aberrant seed (León-Kloosterziel et al., 1994). Together, these two observations demonstrate that although normal Arabidopsis seeds have an external form that resembles the enclosed embryo, the integuments play a critical role in attaining this form.

Different Mechanisms for *SUP* Action Early and Late in Flower Development

Previous reports have shown that one function of the *SUP* gene is to negatively regulate *AP3* in the fourth whorl of floral organs (Schultz et al., 1991; Bowman et al., 1992). Absence of SUP activity in the fourth whorl of *sup* mutants leads to ectopic expression of *AP3* and to persistent expression of *PI* in this same compartment (Bowman et al., 1992; Goto and Meyerowitz, 1994). This ectopic expression appears to be the direct cause of formation of stamens and of stamen/carpel mosaic organs in place of normal carpels in the fourth floral whorl of such mutants (Jack et al., 1994).

The demonstration that one function of *SUP* is suppression of *AP3* expression in the fourth whorl of floral organs led us to question whether ectopic *AP3* expression could also be responsible for the ovule phenotype of *sup* mutants. The detection of *AP3* mRNA in ovules (Jack et al., 1992), more specifically within the outer integument (Modrusan et al., 1994), lends weight to this possibility. The role of *AP3* in manifestation of the Sup$^-$ ovule phenotype was tested through examination of *sup ap3* double mutants. This analysis showed that the Sup$^-$ ovule phenotype occurred independent of the presence or absence of a wild-type *AP3* gene. The *ap3-3* allele used in these experiments appears to be a true null allele (Jack et al., 1992), which demonstrates that ectopic *AP3* expression is not responsible for the effect of *sup* mutations on ovule morphology. Thus, *SUP* must control morphological development of the outer integument through an as yet undetermined mechanism distinct from its previously demonstrated role as a cadastral gene regulating *AP3* expression in the fourth floral whorl.

Additional evidence supports the assertion that *AP3* is not involved in the ovule phenotype of *sup* mutants. The absence of a visible ovule phenotype in *ap3* mutants (Figure 5A; Jack et al., 1992) is inconsistent with hypotheses suggesting that alterations in *AP3* expression patterns could result in the altered shape of Sup$^-$ ovules. Recent experiments have shown that ectopic expression of *AP3*, which is under the control of the cauliflower mosaic virus 35S promoter region, results in phenocopies of *sup* mutants with respect to the fate of the fourth whorl organs (Jack et al., 1994). However, the ovules of such plants are wild type in appearance (B. Krizek and E.M. Meyerowitz, personal communication), supporting the absence of a role for AP3 in ovule development.

The effects of *sup* mutations on ovules appear to be independent of the floral whorl in which ovule-bearing organs form. As noted previously, *ap3* mutants can produce carpels with normal ovules from third whorl primordia (Figure 5A). Similarly, mutations at the *AP2* locus can lead to the formation of carpelloid organs in the first floral whorl (Bowman et al., 1989; Kunst et al., 1989). Although some aberrant ovulelike structures are formed on these carpelloid organs, they also produce normal ovules (Kunst et al., 1989; Modrusan et al., 1994). In contrast, *sup ap3* and *sup ap2* double-mutant plants produce only ovules with Sup$^-$ morphology, regardless of the floral whorl in which the ovules form (Figures 5B and 5C; see also figure 5 of Schultz et al., 1991, and figure 1P of Bowman et al., 1992). These results demonstrate that presence and absence of active *SUP* genes have similar effects on the morphology of ovules whether they form from the surface of ectopic carpels or from normal carpels in the fourth floral whorl. The formation of normal ovules in ectopic carpels indicates that in addition to *SUP*, the other genes necessary for ovule development must also be properly expressed in these structures.

Together, the aforementioned results demonstrate that the mechanism of *SUP* action early in flower development when the fates of the third and fourth whorls are specified must be distinct from the mechanism that governs integument formation late in flower development. We therefore predict that products of the *SUP* gene are present both in the region of the floral apex that gives rise to the fourth whorl of organs (the anlage of the fourth whorl) early in flower development (Schultz et al., 1991; Bowman et al., 1992) and in the abaxial part of the outer integument later in flower development.

Fertility Effect of *sup* Mutations

sup mutants exhibit dramatically reduced seed set as a result of partial female infertility (Table 2; Schultz et al., 1991). Because *sup* mutants have both altered carpels and altered ovules, it was unclear whether both of these effects reduce fertility. Our observation that *sup-5 ap3-3* double mutants have Sup$^-$ ovules but have pistils that are morphologically indistinguishable from those of *ap3-3* single mutants (Figure 5) provides a means to address this question. It was found that the fraction of ovules that went on to develop into seeds in successfully fertilized pistils was significantly lower in *sup-5 ap3-3* double mutants than in *ap3-3* single mutants and was similar to the fraction in *sup-5* single mutants (Table 2). Thus, it appears that the Sup$^-$ ovule phenotype has a significant negative effect on female fertility. Because we did not observe defects in the structure of the embryo sacs of *sup* mutants, we hypothesize that this effect on fertility is the result of a decrease in the ability of pollen tubes to successfully enter the abnormally positioned micropyles of Sup$^-$ ovules.

Whereas the aforementioned results show that the ovule phenotype contributes to the infertility of *sup* mutants, there was also an indication that the aberrant carpel structures may have an effect. It was found that an equal percentage of *ap3-3* and *ap3-3 sup-5* flowers set some seed (as measured by expansion of pistils into siliques) and that this percentage was much greater than that of pistils from *sup-5* single mutants (Table 2). This suggests that a significant subset of pistils (or fused stamen/pistil structures) of *sup* mutants may be defective in providing a suitable avenue for pollen tube access to the ovules. Thus, it appears that both the altered pistil morphology and the lack of normal ovule configuration contribute to the reduced seed set of *sup* mutants.

Phylogenic Considerations

Because of the relative morphological conservation of ovules within taxa, ovule morphology has proven a useful criterion for phylogenetic analyses within and between angiosperm families and in examination of the relationships between angiosperms and other seed plants (for examples, see Crane, 1985; Tobe and Raven, 1987; Taylor, 1991; Doyle et al., 1994). One of the most distinctive features of ovule morphology used in such studies is the location of the micropyle relative to the funiculus. In particular, those ovules with the micropyle adjacent to the funiculus (as in anatropous or amphitropous ovules) are seen as most different from those with the micropyle distal to the insertion of the funiculus (orthotropous ovules). Wild-type ovules of Arabidopsis have the micropyle directly adjacent to the funiculus and have been interpreted as either anatropous when only this feature is considered (Misra, 1962; Webb and Gunning, 1990; Mansfield et al., 1991) or amphitropous if both this feature and the curvature of the embryo sac are taken into account (Robinson-Beers et al., 1992). In contrast, while the ovules still show some curvature as a result of the asymmetry of the outer integument primordium, the micropyle of *sup* mutant ovules is distal to the insertion of the funiculus and therefore represents a largely orthotropous morphology. This demonstrates that loss of a single gene can result in interconversion of these two very different classes of ovules. It is possible that evolutionary changes in *SUP* gene expression or protein function were important components in evolution of the divergent ovule morphologies observed in extant angiosperms. The availability of a clone of the Arabidopsis *SUP* gene would facilitate analysis of the sequence and expression pattern of *SUP* orthologs in other species to further explore this hypothesis. It is interesting that Arabidopsis *ats* mutants produce reproductively competent ovules that have only a single integument (León-Kloosterziel et al., 1994). Thus, it is possible that only two genes differentiate orthotropous, unitegmic ovules from anatropous (or amphitropous), bitegmic ovules in angiosperms.

The difference between the bilaterally symmetrical outer integument of wild-type Arabidopsis plants and the nearly radially symmetrical, tubular outer integument observed in *sup* mutants is reminiscent of the difference between bilaterally symmetrical (zygomorphic) flowers and radially symmetrical (actinomorphic) flowers. In Antirrhinum, mutations of the *cycloidea* locus lead to the formation of largely actinomorphic flowers in this species that normally has highly zygomorphic flowers (Carpenter and Coen, 1990; Lister et al., 1993). This effect is produced in *cycloidea* mutants as a result of the adaxial parts of a flower developing to resemble more closely normal abaxial parts than is the case in wild-type flowers. Thus, both *sup* and *cycloidea* mutations result in a decrease in the differences between the abaxial and adaxial regions of the affected structures. Possibly, a common mechanism is involved in the action of both of these genes. Alternatively, these two systems may provide examples of two independent mechanisms for establishing developmental gradients necessary to allow formation of bilaterally symmetrical structures.

In summation, the *SUP* gene has now been shown to play critical roles in both determination of carpel identity and proper morphological development of ovules. Additional studies on the nature and expression of this gene promise to contribute significantly to our understanding of the regulation of plant development and may provide insight into the evolution of floral form and ovule morphology.

METHODS

Plant Material

superman-5 (*sup-5*) and *inner no outer* (*ino*) mutants were isolated from a screen of M_2 plants derived from ethyl methanesulfonate–mutagenized seed of *Arabidopsis thaliana* ecotype Landsberg *erecta* (Lehle Seeds, Round Rock, TX). The mutant screening procedure was similar to that used for the isolation of *bel1-1* and has been described previously (Robinson-Beers et al., 1992). Briefly, plants derived from M_2 seed were screened for siliques that failed to expand. Plants thus identified were pollinated with wild-type pollen to test for female sterility and were also used to pollinate wild-type flowers to test for male sterility. The *sup-5* and *ino* mutants were backcrossed to the wild type three times, and plants of the mutant phenotype were selected from each segregating generation prior to the analyses described here.

The *apetala3-3* (*ap3-3*) seed used in this study were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The *sup-1* seed were a gift of E.M. Meyerowitz (California Institute of Technology, Pasadena, CA).

Seeds were sown in a 1:1:1 mixture of perlite, fine vermiculite, and peat moss. Plants were grown under continuous fluorescent and incandescent illumination at 22°C and fertilized weekly with a complete nutrient solution (Kranz and Kirchheim, 1987).

Allelic Designation

The *SUPERMAN* locus has been referred to by two different names: *SUPERMAN* (Meyerowitz et al., 1991; Bowman et al., 1992) and *FLORAL MUTANT10* (Schultz et al., 1991). The allelic designations correspond as follows: *sup-1* is *flo10-2*; *sup-2* is *flo10-1*; *sup-3* is *flo10-3* (Bowman et al., 1992). We have arbitrarily chosen to refer to this locus as *SUPERMAN* in this work, and because Bowman et al. (1992) note a total of four prior alleles of *sup*, we have designated the new allele described herein as *sup-5*.

Scanning Electron Microscopy

Pistils were partially dissected and immediately placed in 5% glutaraldehyde in cacodylate buffer (50 mM sodium cacodylate, pH 7.0) and then placed under house vacuum for at least 15 min. Following slow release of the vacuum, the pistils were fixed by storing overnight at 4°C. The fixed specimens were washed four times in cacodylate buffer and postfixed in 2% osmium tetroxide in cacodylate buffer overnight at 4°C. The specimens were again washed four times in cacodylate buffer and dehydrated through a graded alcohol series of 20, 40, 60, 80, 95, and 100% ethanol. Critical point drying was performed overnight in liquid carbon dioxide. Specimens were mounted on stubs, dissected further, sputter coated with gold, and examined with an ISI DS130 scanning electron microscope (Topcon Technologies, Paramus, NJ) at an accelerating voltage of 10 kV.

Light Microscopy

Ovules were cleared using the procedure of Herr (1971). Whole or partially dissected pistils were fixed for 24 hr at 4°C in a 5:5:90 mixture of formalin, propionic acid, and 50% ethanol. The specimens were washed once in clearing solution (85% lactic acid, clove oil, phenol, chloral hydrate, xylene; 2:2:2:2:1 [v/v]) and stored for 24 hr in fresh clearing solution. Pistils were further dissected, mounted on slides, and photographed on a Zeiss (Oberkochen, Germany) Axioplan microscope equipped with differential interference contrast optics.

Double-Mutant Construction

ap3-3 sup-5

Homozygous *sup-5* plants were used to pollinate emasculated homozygous *ap3-3* flowers. F_2 seed was collected from 12 phenotypically wild-type F_1 plants and was sown. Among the resultant F_2 population, a novel phenotypic class with *ap3-3* flowers and *sup-5* ovules was observed. The F_2 progeny segregated 238 wild-type:69 *ap3-3*:76 *sup-5*:16 *ap3-3 sup-5* plants. These data fit a 9:3:3:1 ratio (χ^2 = 4.608, $P > 0.20$).

ino sup-5

Homozygous *ino* plants were used to pollinate *sup-5/SUP* flowers. F_1 seed was collected from six independent crosses and sown, and the F_1 plants were grown to maturity. F_2 seed was collected from five phenotypically wild-type F_1 plants. Among the resultant F_2 population were three families that did not segregate for *sup-5*. Presumably, these families resulted from F_1 plants with an *ino/INO SUP/SUP* genotype. In addition to wild-type and *ino* plants, the other two families segregated both *sup-5* and plants with the novel phenotype of Sup-5$^-$ flowers bearing Ino$^-$ ovules. These two families segregated 58 wild-type:18 *ino*:10 *sup-5*:5 *ino sup-5* plants. These data fit a 9:3:3:1 ratio (χ^2 = 4.069, $P > 0.25$).

bel1-1 sup-5

Homozygous *sup-5* plants were used to pollinate flowers of plants heterozygous for the *bell* mutation (*bel1-1/BEL1-1* flowers). The F_1 seed from four independent crosses was collected and sown. F_2 seed was collected from 12 phenotypically wild-type F_1 plants. Six of the families in the F_2 population did not segregate for *bel1-1*. Presumably, these families arose from F_1 plants that had a *sup-5/SUP BEL1/BEL1* genotype. In addition to wild-type and *sup-5* plants, the other six families segregated for *bel1-1* and plants with the novel phenotype of Sup-5$^-$ flowers bearing Bel1-1$^-$ ovules. These six families segregated 134 wild-type:39 *bel1-1*:32 *sup-5*:14 *bel1-1 sup-5* plants. These data fit a 9:3:3:1 ratio (χ^2 = 3.057, $P > 0.35$).

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